Molecular and cellular neuroplasticity in animal models of depression

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Chapter 4

Behavioral therapy rescues cognitive impairments, reduced hippocampal LTP and increased synaptic AT1B2 expression long after social defeat

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Abstract

Depressed patients often persistently suffer from comorbid cognitive impairments, such as deficits in explicit memory. Here, we modeled persistent affective depression symptoms and memory deficits in a social defeat paradigm in rats. We found that hippocampus-dependent cognitive impairments were still apparent three months after social defeat stress. This maintenance phase of depression was characterized by increased levels of the Na,K ATPase subunit AT1B2 in hippocampal synapses and by a reduced potential to evoke hippocampal CA1 long-term potentiation (LTP). The antidepressant imipramine normalized the level of AT1B2 and rescued the hippocampal LTP deficit and cognitive decline. Most importantly, we found that behavioral therapy, consisting of daily housing in an enriched environment for one hour, thereby modeling positive activities and exercise, was just as effective as imipramine treatment in relieving all molecular, cellular and behavioral aspects of the depression-like state.

Introduction

Major depressive disorder (MDD) is a complex neuropsychiatric syndrome. Although MDD is most markedly typified as a mood disorder, with strong affective symptoms, most MDD patients suffer from associated cognitive impairments, such as disturbances in explicit and spatial memory[16,130]. Thus, MDD has debilitating properties both in the affective and cognitive domains, eliciting questions concerning underlying mechanisms and adequate therapies.

Stress is a potent causal factor in eliciting MDD. Over the years, compelling evidence has linked cognitive deficits in MDD to reduced hippocampal volume[12,154], and impaired hippocampal function[155]. In line with this, rodent models of depression involving acute stress showed morphological changes in the hippocampus, e.g., neuronal atrophy and reduced neurogenesis, plasticity changes, e.g., reduced long-term potentiation (LTP), and stress-induced reduction of dorsal hippocampus-dependent spatial learning (reviewed in[35,156]. However, paradigms with clinical validity, modeling and examining the maintenance phase of depression weeks to months after initial stress exposure, are scarce. To assess the long-term effects of stress exposure on cognitive function, to reveal its underlying synaptic mechanisms, and to investigate treatment options, we adopted a social defeat paradigm in rats, in which five daily encounters of social defeat were followed by individual housing for a period of 12 weeks[33]. This particularly enabled us to model long-term stress effects, as social isolation would appear to be particularly relevant to certain subtypes of human depression[157], and moreover, a combination of active and passive stress has strong precipitating effects on the development of the disease[84]. Importantly, the majority of stress stimuli that lead to MDD are of psychosocial nature[82],

This stress paradigm has been shown to cause insensitivity to rewards[33], resulting in impaired reward anticipation behavior indicative of an anhedonic state, a core symptom of depression, that can be counteracted by chronic antidepressant therapy[141]. In the present study, the long-term effects of social defeat stress on dorsal hippocampus-dependent cognitive performance were addressed. We examined the cellular plasticity mechanisms contributing to these impairments, and we investigated the changes in the synaptic proteome long after social defeat stress exposure. Since in humans, physical exercise and positive psychosocial activities can improve cognitive function, reduce depressive symptoms and increase stress resiliency[158], we questioned whether, next to pharmacotherapy, behavioral therapy would alleviate impairments in the social defeat paradigm at the molecular, cellular and behavioral level.

Materials and methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) 8 weeks of age, weighing 180–200 g at the time of arrival were initially socially housed (2 per cage) in Makrolon type IV cages (Tecniplast, Milan, Italy) in a temperature-controlled room (21±1 °C) under regulated lighting conditions (lights on at 7:00 p.m. and off at 7:00 a.m.). Food and water were available *ad libitum*. Long-Evans male rats (Harlan, UK), weighing 300–350 g were used as residents for social defeat[141]. These animals were pair-housed with age-matched sterilized females in plastic cages (63 x 25 x 33 cm) located in a separate room. Housing conditions were the same as for Wistar rats. All experimental manipulations were conducted during the dark phase (activity period) under a dim red light. All experiments were approved by the Animal Users Care Committee of the VU University Amsterdam.

Experimental design and treatment

Wistar rats (age ≥ 11 weeks) of the social defeat group were subjected to 5 days of social defeat stress and were then housed individually for three months in macrolon class III cages from the first defeat onwards, as described before[140] (Supplemental Fig. 1). Control rats were housed in pairs. The social defeat procedure consisted of daily resident–intruder interaction sessions using dominant male Long-Evans rats for five consecutive days. Control animals were handled daily.

During the last three weeks of this social isolation, rats were treated by gavage administration of the antidepressant imipramine (20 mg/kg per 0.5 ml water; Sigma- Aldrich, Germany), behavioral therapy (BT), consisting of housing in an enriched environment for one hour every day, or water as control. This 2 x 3 design resulted in six experimental groups: (1) control rats with water (Control+H20), (2) control animals with chronic imipramine treatment (Control+IMI), (3) control animals with BT (Control+BT), (4) socially defeated animals with water (Defeat+H20), (5) socially defeated animals with chronic imipramine treatment (Defeat+IMI), and (6) socially defeated animals with BT (Defeat+BT). All behavioral, electrophysiological and molecular analyses were performed at the end of treatment, unless stated otherwise.

Reward anticipatory behavior

A classical Pavlovian conditioning setup was used to investigate anticipatory behavior, as described earlier[140]. To investigate the behavioral response to the conditioning stimulus

(repetitive sound (keyboard) and light flashes (three times)), animals were observed before training (trial 0) to determine baseline activity, and again after 35 training trials of pairing with a 5% sucrose-reward, using the computer program 'The Observer' (Noldus Information Technology, Wageningen, The Netherlands). The researcher who analyzed the behavioral data had no knowledge of the experimental groups. Differences in activity (reflected by frequency or transitions of behavioral elements) displayed before training compared with those after training were used as parameter for reward anticipation.

Sucrose preference

The preference for sucrose (5%) was measured in a two-bottle (sucrose and water) consumption test. Consumption was assessed after 24 h by reweighing the pre-weighted bottles. After 2 days, the consumption test was repeated. In case of social housing, consumption for each subject was set to half of the total consumption. Sucrose preference was expressed as the increase in consumption (gram) relative to water (gram), and this difference was represented as percentage of the total consumption (gram) [100% x (Δ sucrose–water)/total volume sucrose and water consumed].

Recognition tests

Recognition memory testing was conducted based on two previously reported tasks relying on spontaneous exploration of objects in an open field[143,144]. All testing was carried out in a rectangular arena, 79 x 57 in surface with 42 cm tall black walls. The box was surrounded by visual cues: computer light coming from the west side; a white wall (north); a metal rack (east side); and an open space where the experimenter was sitting (south). The arena was always placed inside the room at the same location and in the same orientation. All rats were habituated to the empty arena twice for 30 minutes on the two days preceding behavioral testing, as well as on the test day for 10 min and 1 min prior to the sample phase.

Object-recognition task – Round or square aluminum bars were used as novel or familiar objects and were chosen using a pseudorandom protocol, balanced across treatment groups. In the 4-minute sample phase, rats were exposed to two identical objects (round or square metal bars) followed by a 15 min inter-trial interval (Supplemental Fig. 2). In the test trial, one object (chosen using a pseudorandom protocol, balanced across treatment groups) was replaced with a novel object and rats were allowed to explore both objects for 4 minutes (Supplemental Fig. 2). The familiar object was a third copy of the two objects seen in the sample phase, to prevent possible carry-over of olfactory cues. Each session was recorded by a video camera suspended above the field and interfaced with a computerized

tracking system using the 'Viewer' software package (BIOBSERVE, GmbH, Bonn, Germany). The nose of the rats was tracked by the 'Viewer' software and during both trials, the object exploration (defined as the time of the nose spent within 2 cm from the object) was measured for each object. Only rats that had accumulated at least 15 s of exploration at each object within the sample phase were included in the analysis (all but one tested met criteria). To further analyze object discrimination during the test trial, the discrimination index ((novel-familiar)/(novel + familiar)) was calculated for each rat using the individual object-exploration times recorded.

'Allocentric' object-place task – For the 4-minute sample phase, rats always entered the testing box from the south, and were placed facing the south wall. Rats were exposed to two identical objects (round aluminum bars) followed by a 15 min inter-trial interval. During test trials, rats entered the box from the east or west side and were placed facing the east or west wall, respectively. Thus, for each trial, the entry point on the sample and test phases were different. In the test phase a third and fourth copy of the same object were placed back in the arena: one in a familiar corner and one in a novel corner (Supplemental Fig. 2). The location of the novel object-place configuration was counterbalanced such that on each trial different corners were used as familiar and novel locations. Rats were now only able to discriminate between objects based on their location with respect to its surrounding. Further analysis of discrimination was the same as for the object recognition task.

Long-term potentiation (LTP) measurements

Rats were sacrificed by decapitation. Subsequently, brains were rapidly removed, and placed in ice-cold artificial cerebrospinal fluid (ACSF; in mM: NaCl 124, KCl 3.3, KH_2PO_4 1.2, $MgSO_4$ 1.3, CaCl2 2.5, NaHCO3 20 and Glucose 10.0, constantly gassed with 95% O_2/5% CO_2). Horizontal hippocampal slices were cut on a vibrating microtome at 400 µm thickness and then placed in a submerged-style holding chamber in ACSF, bubbled with carbogen (95% O_2, 5% CO_2). Slices were allowed to recover for 1 hour following slicing. A planar multi-electrode recording setup (MED64 system, Alpha Med Sciences Co., Ltd, Tokyo, Japan) was employed to record the field excitatory post-synaptic potential (fEPSP), and to study LTP as described in[159](see supplemental materials and methods).

iTRAQ-based Proteomics

To analyze differential expression of hippocampal synaptic membrane proteins between experimental groups, quantitative iTRAQ proteomics was performed. To this end, tissue preparation, iTRAQ labeling, two-dimensional liquid chromatography, MS/MS and protein

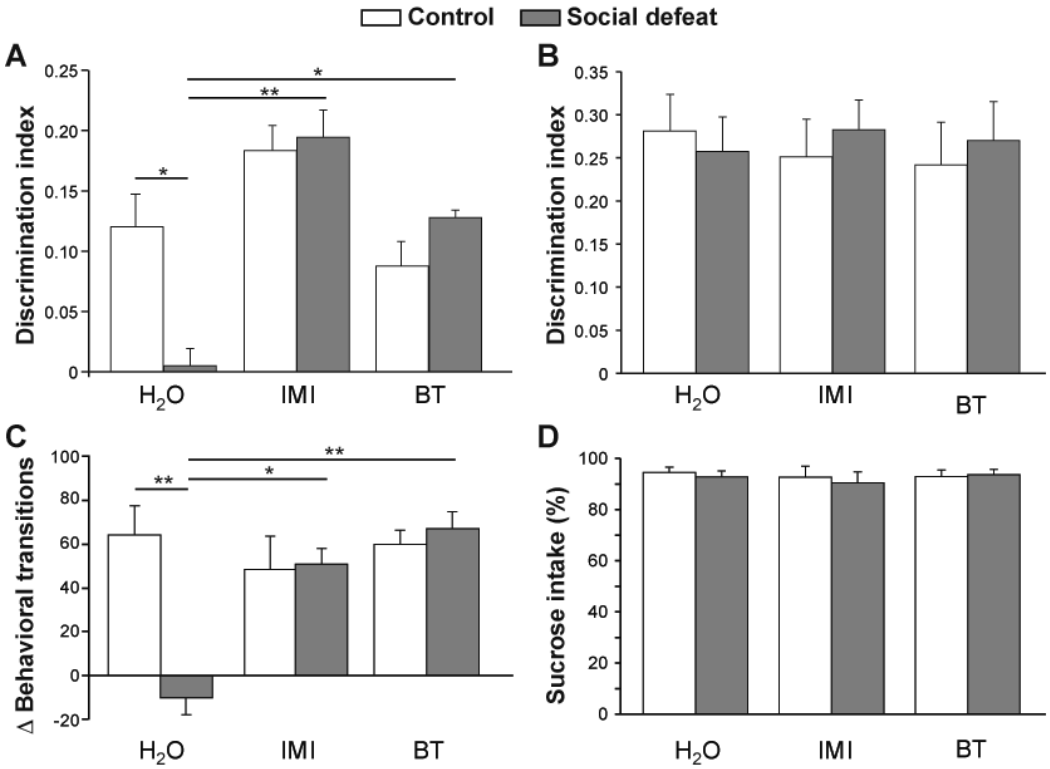


Figure 1. Social defeat impacts on cognitive and affective behavioral tasks. (A) Discrimination index during the test phase of an allocentric object place task. The social defeat paradigm (social defeat) significantly suppressed spatial memory performance, whereas behavioral therapy (BT) and imipramine treatment (IMI) reversed this long-term stress-induced effect (treatment $F(1,47)=5.30$, $P=0.008$;. n=12 for al experimental groups). (B) Discrimination index during the test phase of a novel object recognition task. Social defeat does not affect performance on this task. (C) Anticipation towards 5% sucrose expressed as the difference in activity in the CS-US interval post-training compared with pre-training. Social defeat significantly suppressed reward anticipation, whereas behavioral therapy and imipramine treatment reversed this effect (defeat x treatment interaction $F(1,33)=5.48$, $P=0.0093$). (D) Sucrose preference. Social defeat does not affect sucrose preference (sucrose intake – water intake)/total fluid intake (%). Data is presented as mean ± SEM. LSD *Post hoc*: $*P<0.05$, $**P<0.001$.

identification and quantification were performed as described previously[145,160] (see supplemental materials and methods).

Immunoblotting

The total homogenate and synaptic membranes of the dorsal hippocampus was isolated from an independent group of animals (n=6) as compared to those used in iTRAQ. Samples (3 – 5 μg) were lysed in Laemli lysis buffer, separated by electophoresis on a Criterion 10.5–14% Tris-HCl sodium dodecyl sulfate-polyacrylamide precast gel (Bio Rad Laboratories), and blotted as described before[145]. The following antibodies were used: mouse anti-β-CaM Kinase II (ZYMED Laboratories; 1:1000), mouse anti-Sodium/potassium-transporting ATPase subunit beta-2 (Santa Cruz, 1:5000), mouse anti-Caseine Kinase IIβ (Santa Cruz,

1:1000), mouse anti-cAMP-dependent protein kinase catalytic subunit alpha (PKA) (Santa Cruz 1:1000).

Statistical analysis

The iTRAQ-based proteomics was performed in six biological independent experiments. Proper correction for multiple measurements was carried out in the R computing environment (1.81, Raqua) using the Linear models for Microarray data package[161] (Limma, 1.3.13), which is part of the Bioconductor project[162] (http://www.Bioconductor.org) with adjusted *P*-values[163]. For all other data, statistical analysis was performed using SPSS18.0. All results are expressed as group means ± SEM. Treatment effects were assessed with two-way analysis of variance (ANOVA), followed by Least-square difference (LSD) *post-hoc* analyses.

Results

Social defeat results in hippocampus-dependent cognitive impairments

Using a model of the maintenance phase of depression, in which 5 daily social defeat sessions were followed by individual housing for 12 weeks (Supplemental Fig. 1), we tested recognition memory based on spatial position and novelty of the object[144] (Supplemental Fig. 2). Social defeat decreased performance in the hippocampus-dependent object-place memory task (*P*=0.0034; Fig. 1A). Pharmacotherapy, in the form of imipramine treatment only during the last three weeks of the paradigm (Supplemental Fig. 1) was able to reverse this deficit, illustrating the predictive value of this model as antidepressants can also restore cognitive deficits in depressed patients[63]. Similarly, behavioral therapy (1 h daily enriched housing) was able to completely reverse these cognitive deficits (Fig. 1A), showing the potential of this type of treatment. Pharmacological and behavioral therapies had no effect in control animals. Socially stressed rats showed no difference in object-recognition, a task that is hippocampus-independent. Also there was no effect of treatment in control groups in this task (Fig. 1B). Thus novelty preference and discriminative ability were similar among all groups.

We confirmed that socially defeated rats display a deficit in the affective domain, i.e. an anhedonic phenotype[33,140,141], as shown by a reduced anticipation towards a 5 % sucrose solution compared with control rats (*P*=0.0034). This increase in anhedonia was reversed to control levels by behavioral therapy (Fig. 1C). In addition, a similar reversal was observed

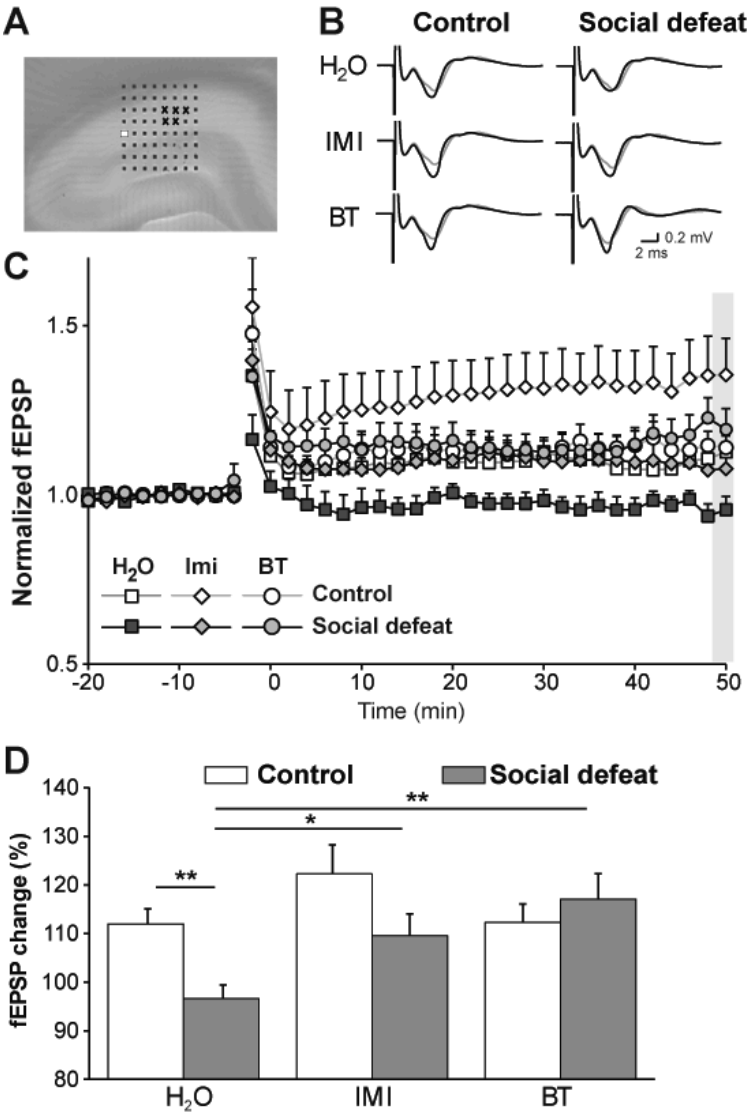


Figure 2. Effect of long-term social stress on LTP in the CA1 of the dorsal hippocampus. (A) Coronal section of the dorsal hippocampus on a MED64 electrode array. An electrode in the Schaffer collateral pathway (white) was used as stimulating electrode. Field potentials were recorded and averaged for the electrodes in the dendritic field of CA1 (marked x). (B) Representative traces from one experiment recorded before (gray) and after (black) LTP induction. (C) Time course of percentage change in fEPSP measured before and after HFS (arrow). (D) Change in fEPSP 50 min after LTP induction (gray indicated in C). Social defeat significantly suppressed CA1 LTP, whereas behavioral therapy (BT) and imipramine treatment (IMI) reversed this effect (defeat: $F(1,45)=4.08$, $P=0.049$; treatment: $F(1,45)=3.96$, $P=0.026$; n=6 for al experimental groups). Data is presented as mean ± SEM. LSD *Post hoc* $^{*}P<0.05$, $^{**}P<0.001$.

for the chronic administration of imipramine[141]. Neither treatment in control animals had an effect on anticipation towards 5 % sucrose. As reported previously, this long-term social defeat paradigm had no significant effect on sucrose preference[164], and no side effects were observed by behavioral therapy or imipramine (all $F(1,30)<0.2$, $P>0.80$, Fig. 1B). Also, no difference in plasma corticosterone levels was observed between any of the groups (all $F(1, 30)<0.2$, $P>0.90$, Supplemental Fig. 3), indicative of the absence of an acute stressor.

Reduction in LTP by long-term social stress is reversed by imipramine and behavioral therapy

As imipramine treatment and behavioral therapy were both capable of rescuing cognitive performance on a hippocampal-dependent memory task, we questioned whether behavioral therapy was able to recover reduced LTP in this long-term social defeat paradigm[140]. Using

a microelectrode array in the Schafer collateral pathway going from CA3 to CA1 subfields of the dorsal hippocampus (Fig. 2A) we applied a tetanus of field potentials. In control rats, average spike amplitude was increased to a response of 113% in CA1 at 50 min after LTP induction (Fig. 2C). LTP was significantly reduced after social defeat (*P*=0.019). This reduced potential to elicit full LTP in socially defeated rats was reversed to control levels by both imipramine and behavioral therapy. Imipramine treatment in controls slightly enhanced LTP, whereas behavioral therapy alone had no side effect on LTP maintenance.

LTP is well-known to be dependent on a the subunit-specific regulation of the AMPARs, during initiation and maintenance phases. In various paradigms, stress has been shown to impact directly on the basal levels of AMPAR subunits, thereby modulating LTP induction and maintenance[43,165,166]. Also, we find that immediately after social defeat AMPAR subunits are regulated (See Chapter 3, Fig. 3). Thus a straightforward explanation of the fact that, long after social defeat stress, LTP induction is impaired is that synaptic AMPAR levels are not at baseline. However, in contrast we find that synaptic expression of the glutamate receptor subunits is normal at this time point (See Chapter 3, Fig. 3). These results prompted us to further investigate the synaptic proteome long after social defeat stress and investigate alternative modes of synaptic modulation.

Reduced spatial performance and CA1 LTP are associated with an increased synaptic expression of AT1B2

To reveal the molecular basis of synaptic plasticity dysfunction that underlies decreased spatial memory after social defeat stress, we compared the proteomes of hippocampal synaptic membrane fractions of socially stressed and control rats. We used iTRAQ labeling of tryptic digests of synaptic proteins, separated these with 2D LC, and identified and quantified peptides using MS/MS (Supplemental Fig. 4). Previously, we used this method to detect subtle changes in synaptic membrane protein abundances in other animal models of disease[145,167]. In total, 382 proteins were identified with at least 3 distinct peptides with a confidence of ≥ 95% present among experimental groups (data not shown). To correct for multiple measurements *P*-values were adjusted to control for the false discovery rate[163]. Five proteins were significantly regulated (adjusted *P*-value <0.05) after social defeat stress (Supplemental Table 1). Differential expression of three of these proteins was confirmed by quantitative immunoblotting in the same sample set (Supplemental Fig. 5, middle panel); Sodium/potassium-transporting ATPase subunit beta-2 (AT1B2), cAMP-dependent protein kinase catalytic subunit alpha (PKAα), and Casein kinase II subunit beta (CSK2B). Of these three proteins AT1B2 was confirmed as upregulated (51%) by social defeat in a biologically

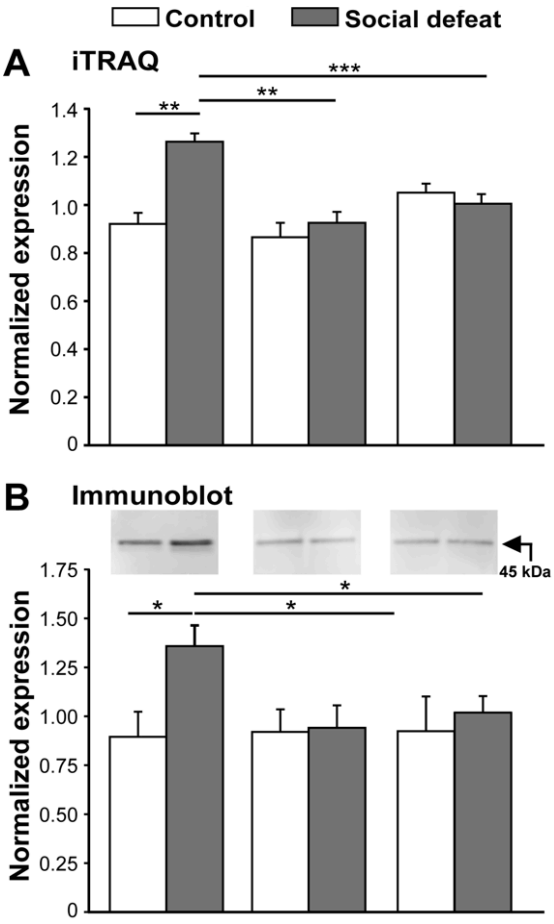


Figure 3. Long-term social stress increases synaptic expression of AT1B2 in the dorsal hippocampus. At the end of treatment, the dorsal hippocampus was dissected for synaptic membrane proteomics analysis. (A) Quantification of iTRAQ reagents revealed a significant higher levels of AT1B2 after long-term social stress (social defeat). Behavioral therapy (BT), and imipramine treatment (IMI) reversed the stress-induced increase in synaptic expression of AT1B2 (stress $F(1,33)=8.05$, $P=0.009$; treatment $F(1,45)=8.58$, $P=0.002$;, stress x treatment interaction $F(1,33)=8.97$, $P=0.001$; see Supplemental Table 1 for adjusted p-values; n=6 for al experimental groups.) (B) Regulated synaptic expression of AT1B2 was validated in an independent set of rats by quantitative immunoblot analysis (stress x treatment interaction: $F(1,45)=4.08$, $P=0.049$; n=8 for al experimental groups). *Insets:* representative example of immunoblots showing the regulated synaptic expression of AT1B2 (45 kDa). Data is presented as mean ± SEM. LSD *Post hoc*: $*P<0.05$, $**P<0.01$, $***P<0.001$.

independent set of animals ($P=0.007$; Fig. 3B, Supplemental Fig. 5, lower panel). At the level of gene expression there was a trend for downregulation of this transcript ($P=0.133$; 16%).

To substantiate that dysregulation of AT1B2 is associated with the animal's depressed state, we measured the effects of both imipramine treatment and behavioral therapy on AT1B2 levels. Indeed, we found that both treatments normalized the increased synaptic expression of AT1B2 after social stress, bringing it back to basal levels (Fig. 3A,B), with no additional effect of treatment alone.

Discussion

Cognitive deficits associated with human depression have been well-characterized[168], with reductions in hippocampus-dependent declarative and spatial memory[16,130]. Recently a link between these reported cognitive impairments and hippocampal function was shown[155], giving way to a hippocampus-dependent symptomatology of depression. In the present study, we showed that in a preclinical model of the maintenance phase of depression, impairments in the affective and cognitive domains are persisting over a long period of time. Because depressed patients still experience symptoms long after the cessation of stress

exposure[82], the long-term social defeat model has a high level of both etiological- and face-validity. Behavioral therapy was able to restore the social defeat-induced deficits from the molecular level all the way up to behavior, similar as achieved by pharmacotherapy using the tricyclic agent imipramine. This indicates that apart from having predictive validity, the present model might also have construct validity, i.e., at the level of the mechanism underlying cognitive core symptoms of MDD.

The reduction in hippocampus dependent memory long after social defeat was found associated with a reduction in the capacity to elicit hippocampal CA1 LTP. This form of synaptic plasticity is known to involve plasticity of glutamate receptors. On the short term, stress-induced effects on LTP are dependent on NMDA receptors[133] and insertion of AMPA receptors[165,169]. Also, stress hormones have been shown to regulate the mobility and postsynaptic membrane levels of AMPA receptors[135]. Accordingly, immediately after social defeat stress glutamate receptor levels are altered. However, here we show that the social defeat-induced impairment of LTP on the long term is not caused by pre-altered synaptic expression of glutamate receptor subunits, as their levels are not affected long after social defeat. Instead, our proteomics analysis showed the increased synaptic expression of a Na,K-ATPase subunit AT1B2 that might well influence the induction of LTP. Importantly, the reversal of AT1B2 to normal levels due to imipramine or behavioral therapy indicates that dysregulation of AT1B2 is indeed related to the disease phenotype. Taken together, our data suggests that on the long-term, different synaptic mechanisms are involved in causing LTP impairments when compared to acute stress.

One might wonder whether the observed changes in the cognitive domain are a consequence of individual housing per se, because social isolation is considered a social stressor by itself[83]. However, at the end of the social defeat paradigm, stress hormone levels have normalized, indicative of the absence of acute stressors. Also, the social defeat-induced changes in basal physiology, i.e. body weight and food/water intake, have already returned to basal levels during the isolation period (Supplemental Fig. 6). Moreover, we showed that hippocampal CA1 LTP is not affected by individual housing without previous social defeat (Supplemental Fig. 7). Thus individual housing after social defeat apparently does not result in a severe stress experience that affects basal physiology and neuronal plasticity.

The observation that reduced CA1 LTP is associated with increased synaptic expression of AT1B2 has interesting implications for the molecular mechanism underling the cognitive deficits observed after social defeat. AT1B2 is a β subunit of the P-type Na,K ATPase family. The protein complex is a heterodimer composed of two subunits: the

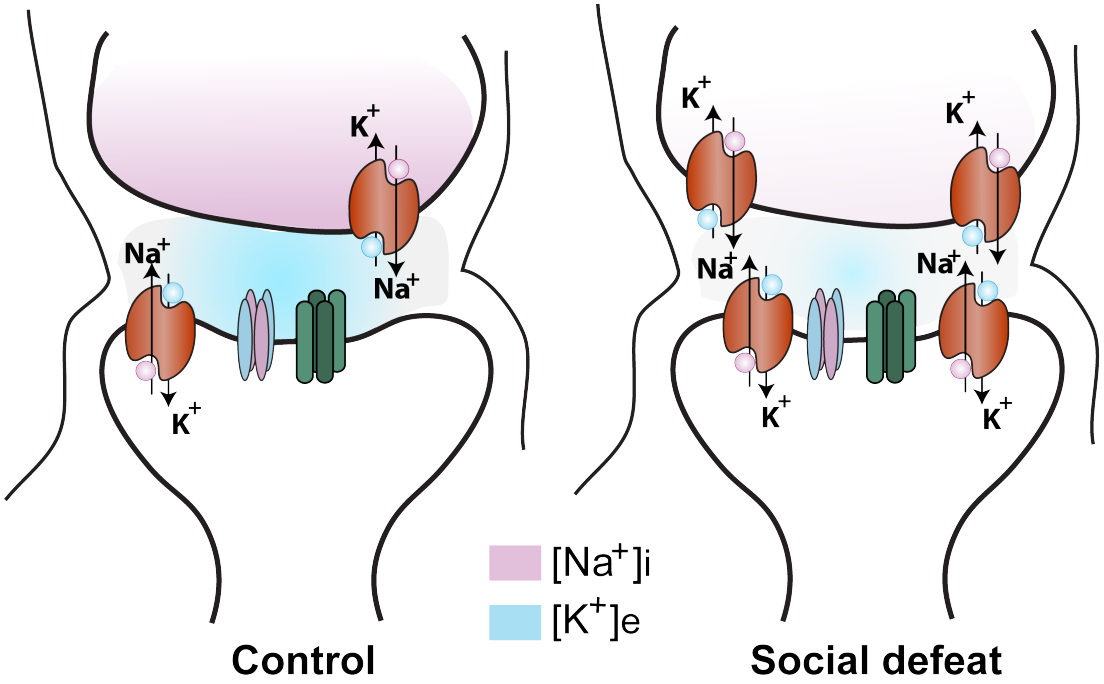


Figure 4. Model of the hippocampal synaptic adaptation after long-term social defeat stress associated with reduced LTP and impaired spatial memory. After social defeat stress and subsequent individual housing, the synaptic levels of subunit AT1B2 of the Na^+/K^+ transporter is increased by ~50%. This increased expression likely causes an increased transporter activity (shown by an increased number of ion pumps), thereby faster clearance of potassium from the synaptic cleft ($[Na^+]_i$), and of sodium from the synaptic compartments ($[K^+]_i$). This causes faster membrane re-polarization after firing of an action potential, and therefore less glutamate release and calcium influx. Together, increased levels of Na^+/K^+ transporter causes less plasticity in hippocampal synapses that might underlie impaired LTP.

catalytic α subunit that contains ATPase activity and the β subunit that regulates, through assembly of alpha/beta heterodimers, the number of sodium pumps transported to the plasma membrane[170,171]. Na,K ATPases are expressed throughout neurons, i.e., at neuronal cell bodies and dendrites, and are found enriched in synaptosomes[172]. They are membrane localized transporters responsible for active transport of Na^+ and K^+ ions across the plasma membrane, thereby generating a gradient responsible for the neuronal rest membrane potential, but also its repolarization after eliciting action potentials[173]. Increased levels of the regulatory subunit AT1B2 after social defeat on the presynaptic site, and hence increased numbers of Na^+/K^+ transporters, will lead to a faster clearance of Na^+ ions from the synaptic spine and influx of K^+ ions from the synaptic cleft (Fig. 4). Consequently, after generation of an action potential, increased pump activity will induce a faster repolarization, resulting in less glutamate release from the presynaptic site and longer after-hyperpolarization[174]. As a result, there will be less activation of postsynaptic glutamate receptors and thus less

induction of plasticity postsynaptically, reflected by reduced LTP. In line with presynaptic effects, increased numbers of Na^+/K^+ transporter post-synaptically will reduce building up a membrane depolarization, counteracting postsynaptic plasticity. The involvement of the Na^+/K^+ transporter in synaptic plasticity has been demonstrated in Leech and Drosophila[170,174,175]

Other subunits of the Na,K ATPase complex, e.g., AT1A1 subunits, have been implicated in antidepressant response, i.e., the effect of lithium[176] and in stress-induced animal models of depression[177]. In this perspective it is noteworthy that a trend of higher synaptic expression has been found for other subunits of this complex, e.g., AT1A2 (*P*=0.095), AT1b1 (*P*=0.170; Supplemental Table 2). Also, most of these subunits show decreased levels of expression due to behavioral therapy, and to a lesser extent due to imipramine, in socially defeated animals.

AT1B2 has been found regulated at the level of gene expression both in clinical and preclinical studies. Lower levels of AT1B2 transcript were observed in the hippocampus of depressed individuals[178], as well as in the hippocampus of a genetic model of endogenous depression, i.e., the Wistar-Kyoto strains[179]), similar to the lower transcript levels observed in this study. However, we find the AT1B2 protein at higher levels in the synaptic membrane fraction, indicating that caution should be taken when translating gene expression data into synaptic protein levels and further into a clinical perspective.

Our finding that the beneficial effects on cognitive domains of both chronic imipramine treatment and behavioral therapy are associated with a recovery to normal levels of synaptic AT1B2 holds important promises for intervention at Na,K ATPase-related mechanisms in the maintenance of depressive symptoms and their treatment. Obviously, application by pharmacological intervention at Na,K ATPases is complicated considering that after social defeat its expression was found only increased at synaptic sites and not in total cell lysates. Moreover, its ubiquitous expression throughout the body further precludes direct pharmacological interference. Fine-tuning the level of these transporters might be crucial, as too low levels could induce anxiety symptoms and increases stress-induced memory impairments in mice[177].

Behavioral therapy, consisting of housing in an enriched environment for one hour daily, thereby modeling positive activities and exercise, was effective in restoring stress-induced long-lasting cognitive impairments and its underlying cellular and molecular deficiencies. Also, in the affective domain, behavioral therapy restored the social defeat-induced reduction in reward anticipation (anhedonia). These findings, when translated to the human condition have important implications for treatment strategies in the clinic. Several forms of

psychotherapy –in particular, cognitive and behavioral therapies– were shown effective for patients with mild to moderate MDD[180], and the combination of medication and psychotherapy can exert a synergistic effect. Our study implies that, in addition to pharmacological treatment, with often bearing negative side effects, another form of behavioral therapy, i.e. physical exercise, should be explored further as treatment option for depressed patients with cognitive impairments and anhedonia.

Supplemental material

Material and methods

Social isolation

Wistar rats (age ≥ 11 weeks) of the social Isolation group were housed individually for three months in macrolon class III cages. Control rats were housed in pairs.

Corticosterone assay

Trunk blood samples were collected via decapitation between 9:00 am and 11:00 am. Samples were collected into a 7-mL heparin-coated tube (Greiner Bio-One, Monroe, North Carolina) and kept on ice. The samples were spun at 1000 x *g* for 10 min. Plasma was decanted and stored at −80 °C until the assay was used. Levels of serum corticosterone were assessed using a rat Glucocorticoid (GC) ELISA kit (Cusabio Biotech Co., LTD), according to the manufacturers instructions.

LTP recording

A planar multi-electrode recording setup (MED64 system, Alpha Med Sciences Co., Ltd, Tokyo, Japan) was employed to record the field excitatory post-synaptic potential (fEPSP), and to study LTP. The methodology has been described in detail elsewhere [159]. Briefly, hippocampal slices were placed on special probes that were fabricated with 8 x 8 electrode arrays and pre-coated with polyethylenimine (PEI, Sigma). P210A probes (Alpha Med Sciences) with an inter-electrode distance of 100 µm were used. Correct placement of the electrodes at the CA3–CA1 region was done manually, monitored by a microscope (MIC-D, Olympus Ltd., Japan) (fig. 2A). Four slices per rat were studied simultaneously. Each slice was superfused by 100 ml oxygenated ACSF, which was recirculated at a flow rate of 2 ml/min. fEPSPs were recorded from multiple electrodes in the dendritic layer of CA1 neurons by choosing an electrode in the Schaffer collateral pathway as the stimulating electrode. Based on the stimulus–response curve, we chose a stimulation intensity that evoked the fEPSP with a magnitude of 50% of the maximum response (around 1 mV in most cases). We found that this setting was suitable for the induction of LTP in healthy slices in the setup. After allowing a stable baseline of 20 min, an induction protocol that evoked LTP was applied, which consisted of 2 trains of 100 Hz stimulus that lasted for 1 s, separated by 10 seconds. The field potential response was recorded for 1 h after the tetanus. LTP was quantified as % change in the average amplitude of the fEPSP taken from 50 to 60 min interval after LTP induction. LTP in al electrodes within the CA1 region was averaged, and average LTP of all 4 slices was defined as final LTP per individual rat.

iTRAQ-based Proteomics

Tissue preparation – Following decapitation, brains were removed and rapidly frozen in ice-cold isopentane and stored at –80 °C until further use. The dorsal hippocampus (Bregma –2.56 till –5.30)

was removed freehand at –20 °C from 1-mm-thick slices. Synaptic membrane fractions were isolated for every hippocampus (left and right pooled). Samples were homogenized in ice-cold 0.32 M sucrose (5% of homogenate was collected as total cell lysate) and then centrifuged at 1000 g for 10 min. The supernatant was loaded on top of a sucrose gradient consisting of 0.8 and 1.2 M sucrose. After centrifugation at 100,000x g for 2 h, the synaptosome fraction at the interface of 0.85⁄1.2 M sucrose was collected and then lysed in hypotonic solution. The resulting synaptic membrane fraction was recovered by centrifugation using the sucrose step gradient as stated above. The synaptic membrane fraction was collected from the 0.85⁄1.2 M interface and protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA). For each sample, 75 μg of protein was used for iTRAQ labeling (see below), and synaptic membrane fractions were dried in a SpeedVac overnight.

iTRAQ labeling – Synaptic membranes were resuspended in 28 μL of dissolution buffer and 2 μL of cleavage reagent [iTRAQ reagent kit, with 0.85% RapiGest (Waters Associates, Milford, MA, USA)] to solubilize synaptic membranes. After incubation for 1 h, 1 μL of cys blocking buffer (Applied Biosystems, Carlsbad, CA, USA) was added and vortexed for 20 min. Next, 10 μL of trypsin (Promega) dissolved in water was added and incubated overnight at 37 °C. Trypsinized peptides from each experimental group were then tagged with iTRAQ reagents (113, CON+H_2O; 114, CON+IMI; 115, CON+BT; 116, Stress+H2O; 117, Stress+IMI; 118, Stress+BT) dissolved in 80 μL ethanol. After incubation for 3 h, the six samples were pooled and acidified with 10% trifluoroacetic acid (TFA) to pH 2.5 – 3.0. After 1 h, the final sample was centrifuged and the supernatant dried overnight in a SpeedVac.

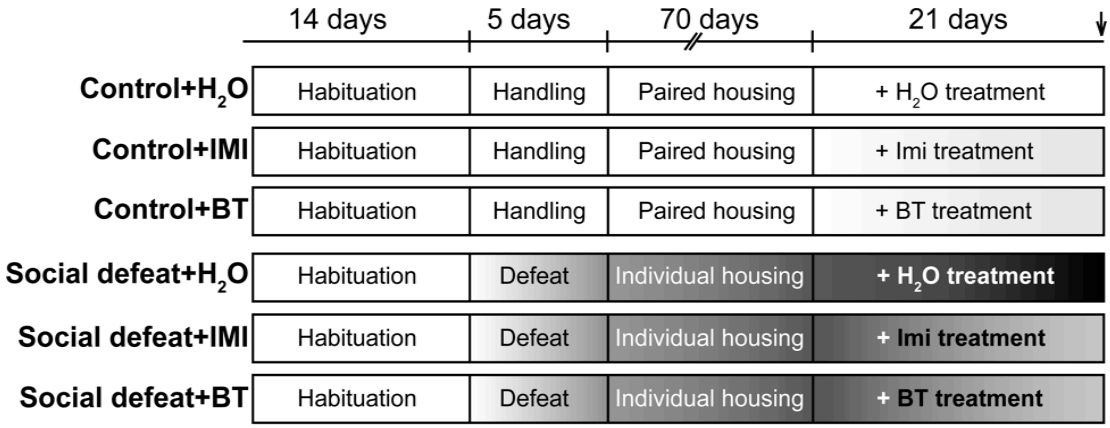
Two-dimensional liquid chromatography – The dried iTRAQ sample was dissolved in 300 μL of loading buffer (20% acetonitrile, 10 mM KH2PO4, pH 2.9) and loaded onto a polysulfoethyl A column (PolyLC, Columbia, MD, USA). Peptides were eluted with a linear gradient of 0–500 mM KCl in 20% acetonitrile, 10 mM KH2PO4, pH 2.9, over 25 min at a flow rate of 50 μL⁄min. Fractions were collected at 1-min intervals. In the second-dimensional liquid chromatography separation, peptides were delivered with a Famos autosampler (Dionex Corp., Sunnyvale, CA) at 30 μL⁄min to a C18 trap column (1 mm x 300 μm i.d. column) and separated on an analytical capillary C18 column (150 mm x 100 μm i.d. column) at 400 nL⁄min using the LC-Packing Ultimate system. Peptides were separated using a linearly increasing concentration of acetonitrile from 5 to 50% in 45 min, and to 90% in 5 min. The eluent was mixed with matrix (7 mg a-cyano-hydroxycinnaminic acid in 1 mL of 50% acetonitrile, 0.1% TFA, 10 mM dicitrate ammonium) delivered at a flow rate of 1.5 μL⁄min and deposited off-line to the Applied Biosystems metal target every 15 s for a total of 192 spots using a robot (Dionex, Sunnyvale, CA, USA).

Mass spectrometry – The Matrix-assisted laser desorption/ionization (MALDI) plates were analyzed on a 4800 proteomics analyzer (Applied Biosystems) and peptide collision induced dissociation (CID) was performed at 2 kV with nitrogen collision gas. MS⁄MS spectra were collected from 2500 laser shots. Peptides with a signal-to-noise ratio above 50 at the MS mode were selected

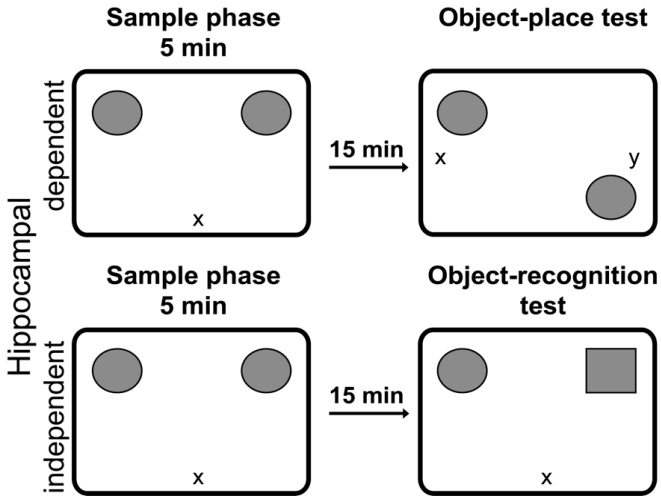
for MS/MS, at a maximum of 25 MS/MS per spot. The precursor mass window was set at 200 relative resolution full width at half maximum (FWHM).

Protein identification – The MS/MS spectra were searched against the rat database [Swissprot and National Center for Biotechnology Information (NCBI)] using GPS Explorer (Applied Biosystems) and Mascot (MatrixScience, Boston, MA, USA). A library was then generated containing all annotated peptides with a confidence interval score higher than 20%. Database redundancy and sequence redundancy were removed. Hence, quantification was performed only on those peptides that were annotated to a single protein, and are referred to as 'unique peptides'. Only proteins identified with ≥ 2 unique peptides, and of which at least one peptide had a confidence interval ≥ 95%, were selected for quantification and statistical analysis. All regulated proteins were analyzed with a large number of peptides, of which the majority was analyzed with high confidence.

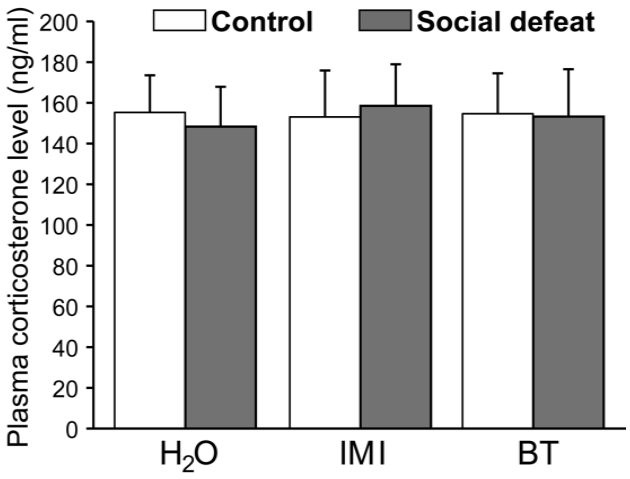
Protein quantification – The iTRAQ experiment was repeated six times with independent samples. The iTRAQ areas (*m/z* 113–118) were extracted from raw spectra and corrected for isotopic overlap using GPS Explorer. To compensate for the possible variations in the starting amounts of the samples, the individual peak areas of each iTRAQ signature peak were $\log_2$-transformed to obtain a normal distribution, normalized to the mean peak area for each sample, and finally standardized to the normalized peak average per peptide. The protein abundances in every experimental group were determined by taking the average normalized standardized iTRAQ peak area of all unique peptides annotated to a protein. In total, the iTRAQ-proteomics experiment was repeated six times with independent biological samples. Only proteins that were identified in five out of six replicates were included for further statistical analyses. For presentation of differential expression of synaptic proteins between groups, regulation was calculated by subtracting the average $\log_2$-transformed protein abundance of groups of interest. For presentation of absolute expression differences, exponents of these values were calculated.



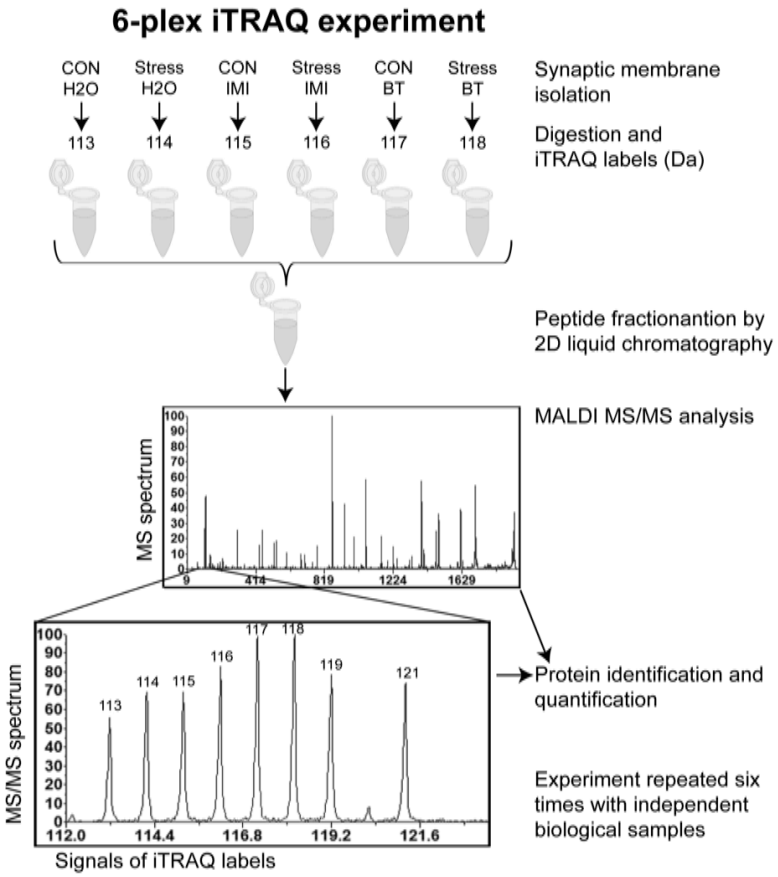
Supplemental figure 1. Experimental design and treatment groups. The social defeat paradigm (social defeat) in combination with behavioral therapy (BT) or imipramine treatment (IMI) was applied in 11-week old rats. After habituation to the new housing conditions, rats of the social defeat group received daily bouts (5 min) of social defeat during 5 days and subsequent individual housing (3 months). Control animals were handled daily for 5 days and were housed in pairs. Behavioral or pharmacotherapy was applied only during the last three weeks (or no treatment, H_2O) of the individual housing period. The length of each period is indicated. All behavioral and electrophysiological and biochemical analysis were performed at the end of the paradigm, at the end of treatment (arrow). Independent cohorts of animals were used for behavioral physiological and biochemical analyses.



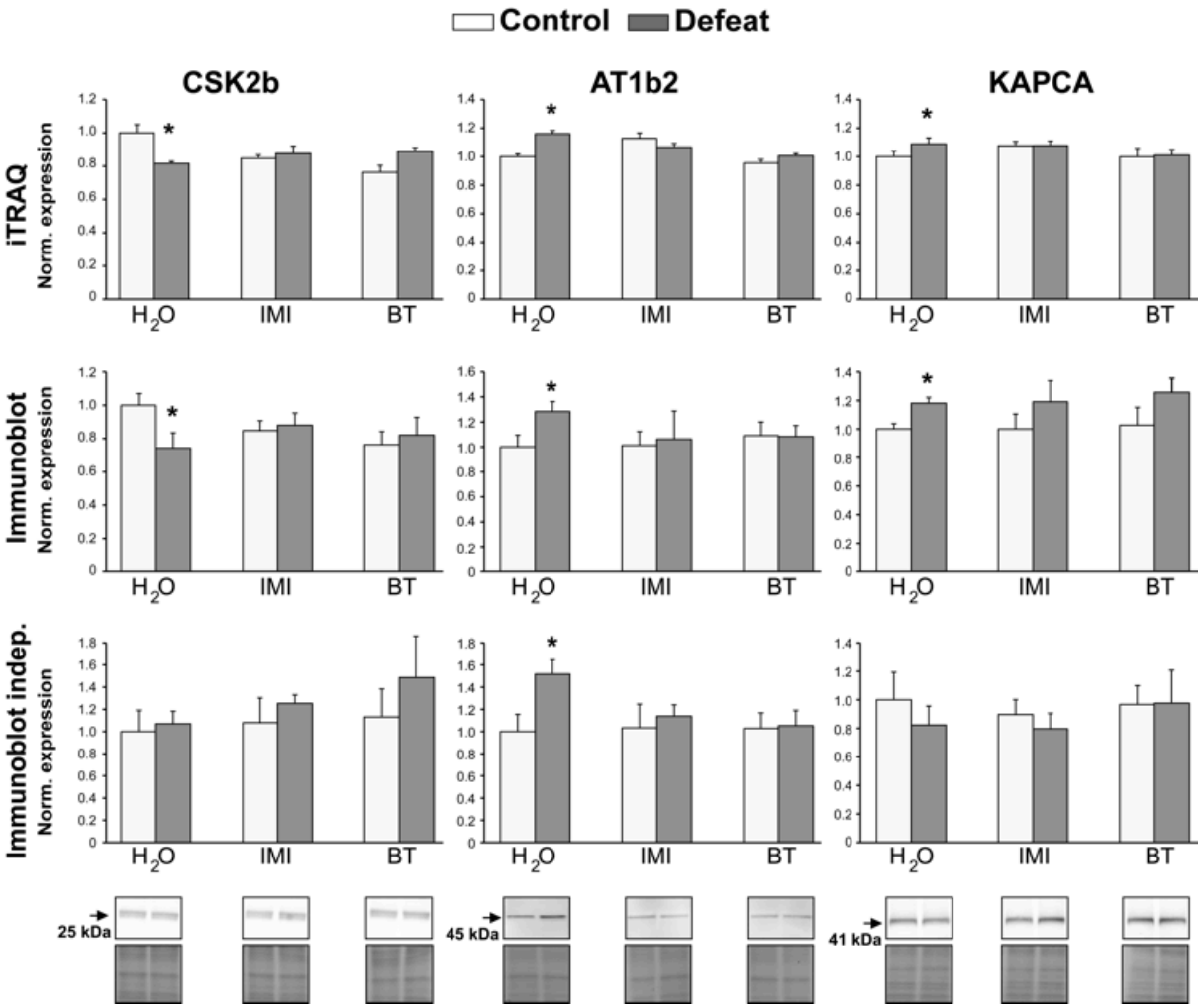
Supplemental figure 2. Experimental design for object-place and object-recognition tests. Schematic representation of the recognition tasks (Place, A; Recognition, B) and geometric arrangement of objects. The 'x' and 'y' indicate start locations of the rat in the tasks. In the object place test the start locations are counterbalanced between 'x' and 'y' over treatment groups. The circle and square represent the different objects.



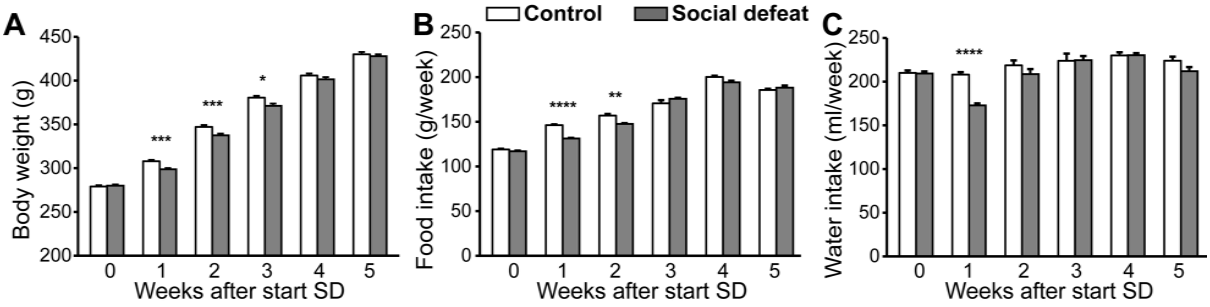
Supplemental figure 3. Mean levels of plasma corticosterone (± SEM), measured at the end of water (H_2O), imipramine (IMI) or behavioral therapy (BT) treatment. No difference between any of the groups was observed.



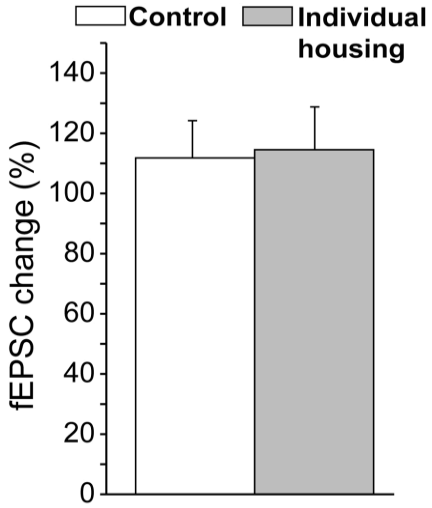
Supplemental figure 4. Set up of the iTRAQ experiments. Synaptic membrane fractions were isolated from one dorsal hippocampus (left and right) from one rat per experimental group. Tryptic digests of synaptic membranes from these samples were tagged with 6-plex iTRAQ reagents (one per experimental group). Peptides from each set of iTRAQ experiment were pooled together, fractionated by two-dimensional liquid chromatography and subjected to tandem mass spectrometric analysis. Protein identification and quantification were performed as detailed in the main text. The experiment was performed six times, each time with a biological independent set of samples.



Supplemental figure 5. **Synaptic expression of CSK2b, AT1b2 and KAPCA determined by iTraq and Western blot.** Protein expression in synaptic membrane samples was determined both by iTRAQ quantitative proteomics (upper panels), and by immunoblot (middle panels) on the same set of samples. Expression differences of these three proteins was tested in an independent set (indep.) by immunoblot (lower panels). Bar graphs show standardized average relative expression levels (vs. Control+H_2O) (±SEM) for CSK2b, AT1b2, and KAPCA (*cf.* Supplemental table 1). *P<0.05 *vs.* Control+H_2O. Regulated expression of only AT1b2 could be validated in the independent set of samples. Insets show representative examples of the immuno-detected band, as well as the coomassie-stained gel, used for correction of input material.



Supplemental figure 6. Basal physiological parameters tested *acutely* after social defeat and their normalization over time. Parameters were acquired in the first five weeks after the start of social defeat, before behavioral therapy and imipramine treatment had started. At this time point only two groups were present: social defeat, and controls. (A) Social defeat reduced body weight (A), food intake (B), and water intake (C). These parameters were normalized after 4, 3, and 2 weeks, respectively. Data is presented as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplemental figure 7. Effect of individual housing on LTP in the CA1 of the dorsal hippocampus. Average change (±SEM) in fEPSP 50 min after LTP induction. Individual housing had no effect on LTP.

Supplemental table 1. Synaptic protein changes after long-term social stress. Five proteins were significantly regulated (adjusted p-value < 0.05) after long-term social stress after adjustment for multiple hypothesis-testing using the Benjamini and Hochberg method. Expression of two of these proteins (AT1B2 and ANXA6) were rescued to control levels by imipramine treatment and three (AT1B2, KAPCA, and ANXA6) were rescued to control levels by behavioral therapy. Regulated expression of three of these proteins (CSK2B, AT1B2 and KAPCA; bold) could be confirmed by quantitative immunoblot analysis on the same samples as used for iTRAQ analysis. Regulated expression of AT1B2 (bold) could be confirmed by immunoblot in an independent set of biological samples. Indicated are protein accession number, protein name, average number of peptides (over six replicate experiments) used for quantification and identification, expression ratios (log2) of Stress+H_2O vs CON+H_2O and their adjusted-p-value for the iTraq data, of Stress+IMI vs CON+H_2O and Stress+BT vs CON+H_2O as a measure of rescue to control levels, of Stress+H2O vs CON+H_2O and their p-values (t-test) of the immunoblots on the samples used for iTRAQ quantification and on an independent set of biological replicates. NA: not available; no antibody suitable for quantification was available.

		iTRAQ					Western blot			
SwissProt Accession number	**Protein name**	**Nr unique peptides**	**Regulation SDH vs. CONH**	**Adjusted P-value**	**Regulation SDI vs. CONH**	**Regulation SDB vs. CONH**	**Regulation SDH vs. CONH**	**P-value**	**Regulation SDH vs. CONH independent**	**P-value indep.**
CSK2B_RAT	Casein kinase II subunit beta	4	-0.29	0.008	-0.19	-0.17	**-0.47**	**0.047**	0.21	0.557
AQP4_RAT	Aquaporin-4	6	0.30	0.008	0.23	0.17	NA	NA	NA	NA
AT1B2_RAT	Sodium/potassium-transporting ATPase subunit beta-2	8	0.21	0.008	0.09	0.01	**0.38**	**0.045**	**0.68**	**0.040**
KAPCA_RAT	cAMP-dependent protein kinase catalytic subunit alpha	7	0.12	0.049	0.11	0.01	**0.24**	**0.009**	0.00	1.00
ANXA6_RAT	Annexin A6	43	0.23	0.014	-0.08	-0.05	NA	NA	NA	NA

Supplemental Table 2. Several Na,K ATPase subunits are rescued by behavioral and pharmacological therapy in social defeated (SD) rats. Proteins as identified by iTRAQ proteomics are indicated by the protein accession number (SwissProt). Comparisons (regulation (log2), *P*-value) are made for the effect of water-treated social defeat (SD_H) vs control (Con_H) animals (upregulation), and the effect of behavioral therapy (BT) or imipramine (Imi) in defeated animals vs. defeated water-treated (SD_H) animals (down-regulation). Orange: *P*<0.05; yellow: *P*<0.1. Note that most of the ATPAse type 1 subunits are affected similarly.

	SD_H *vs.* Con_H		**SD_BT *vs.* SD_H**		**SD_Imi *vs.* Sd_H**	
Protein accession	**Regulation**	**p-value**	**Regulation**	**p-value**	**Regulation**	**p-value**
AT1A1_RAT	0.01	0.769	-0.09	0.127	-0.03	0.473
AT1A2_RAT	0.51	0.095	-0.60	0.015	-0.53	0.072
AT1A3_RAT	-0.16	0.498	-0.14	0.024	-0.13	0.056
AT1B1_RAT	0.09	0.170	-0.19	0.016	-0.11	0.120
AT1B3_RAT	0.08	0.372	-0.16	0.014	0.10	0.093
AT2A2_RAT	0.05	0.293	-0.10	0.045	-0.04	0.343
AT2B1_RAT	-0.23	0.790	-0.07	0.321	-0.04	0.573
AT2B2_RAT	0.04	0.460	-0.08	0.127	-0.03	0.376
AT2B3_RAT	0.01	0.825	-0.11	0.106	-0.04	0.410